Materials and Methods
MATERIALS AND METHODS

The third chapter deals with the materials used and the methodology followed to reach the aim. Both the materials and methods are explained together.

3.1 Materials
3.1.1 Chemicals and instruments

All the chemicals were of analytical grade from Sigma Chemicals and Co (St. Louis, MO, USA), Merck (Darmstadt, Germany) and HiMedia Laboratories (Mumbai, Maharashtra, India) unless otherwise specified. The water used for the analysis was treated by arium 67316 reverse osmosis (Sartorius Stedim Biotech GmbH, Germany). All the spectrophotometric measurements were done using UV 100 (Cyberlab, USA).

3.1.2 Collection of legume seeds

The seeds of *A. auriculiformis* were collected from a nursery in Shimoga, Karnataka, India and *P. roxburghii* were collected from local market of Chumukedima, Dimapur, Nagaland, India during the month of June and April 2009 respectively as a single lot. They were cleaned by removing foreign particles, broken and damaged seeds. In addition, *A. auriculiformis* seeds were separated from the arils attached to them. Both the seeds were dried at 40 °C and stored in air tight containers for further analysis.

3.2 Physico-chemical properties
3.2.1 Determination of physical properties of legume seeds

The following physical properties were determined for both the legume seeds as per Sood *et al.* (2002).

3.2.1.1 Seed weight and dimensions

The color of both the seeds was determined subjectively. The seeds (n=30) were examined for their weight using digital balance (to the nearest 0.001 g) (CPA224S, Sartorious, Germany). The same seeds were measured for their dimensions (cm) such as length, width and thickness using vernier caliper. Proportions of the seed coat and cotyledon were determined.
3.2.1.2 Seed density and seed volume

One hundred seeds of known weight were placed in a measuring cylinder containing 100 mL of distilled water. The volume of water increased was measured and the seed density was calculated as,

\[
\text{Seed density (g/mL)} = \frac{\text{Weight of seeds}}{\text{Volume increased}}
\]

From the same data i.e., increased water volume, the seed volume (mL/100 seeds) was also calculated.

3.2.1.3 Hydration capacity and hydration index

One hundred seeds of known weight were soaked in 200 mL of distilled water in a lightly stoppered flask and left overnight at room temperature. On the next day the seeds were drained, superfluous water was removed with absorbent paper and the swollen seeds were reweighed. Hydration capacity and hydration index was calculated as,

\[
\text{Hydration capacity (g/seed)} = \frac{W_2 - W_1}{\text{Number of seeds}}
\]

Where, \(W_1\) - Weight of unsoaked seeds; \(W_2\) - Weight of soaked seeds

\[
\text{Hydration index} = \frac{\text{Hydration capacity/seed}}{\text{Weight of one seed}}
\]

3.2.1.4 Swelling capacity and swelling index

One hundred seeds of known weight were placed in a measuring cylinder and the seed volume \((V_1)\) was measured. The seeds were soaked in 200 mL of distilled water and left overnight. Seeds were drained out of the water and volume of soaked seeds \((V_2)\) was measured. Swelling capacity and swelling index was calculated as,

\[
\text{Swelling capacity (mL/seed)} = \frac{V_2 - V_1}{\text{Number of seeds}}
\]

Where, \(V_1\) - Volume of unsoaked seeds; \(V_2\) - Volume of soaked seeds

\[
\text{Swelling index} = \frac{\text{Swelling capacity/seed}}{\text{Seed volume (mL)}}
\]
3.2.2 Processing regimens

Both the seeds were randomly divided into 7 batches (200 g/batch). The first batch was kept as raw without any treatments. In case of *A. auriculiformis* whole seeds were kept as raw and in *P. roxburghii*, kernels were kept as raw. Various processing methods adopted for both the legume seeds were summarized in Table 3.1. Before adopting the processing methods, *A. auriculiformis* seeds were subjected to mechanical damage using mortar and pestle made of stone (to enhance water absorption over their hard, shiny seed coat for the moist cooking methods).

**Table 3.1 Processing regimens of A. auriculiformis and P. roxburghii seeds**

<table>
<thead>
<tr>
<th>S.No</th>
<th>Treatment mode</th>
<th>A. auriculiformis</th>
<th>P. roxburghii</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Moist cooking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>Soaking followed by autoclaving</td>
<td>Soaking medium</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2% Ash</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1% Sodium bicarbonate</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1% Sugar</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Distilled water</td>
<td>✓</td>
</tr>
<tr>
<td>1b</td>
<td>Autoclaving without soaking</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>1c</td>
<td>Natural fermentation†</td>
<td></td>
<td>✗</td>
</tr>
<tr>
<td>2</td>
<td>Dry cooking</td>
<td>Dry heating</td>
<td>✓</td>
</tr>
</tbody>
</table>

* - Soaking/16 h; ** - Soaking/12 h; ✓ – autoclaving/20 min; ✓!! – autoclaving/15 min; † – autoclaving/25 min; † † – Natural fermentation at room temperature/36 h; † † † – Dry heating at 105 °C/5 min; † † † † – Dry heating at 130 °C/20 min.

3.2.2.1 Soaking followed by autoclaving

Both the legumes seeds of II\textsuperscript{nd} - V\textsuperscript{th} batch were soaked in following soaking solutions in the ratio of 1:10 (seed:soaking solution, w/v) and kept for 16 and 12 h for *A. auriculiformis* and *P. roxburghii* respectively. Batch II-0.2% ash (prepared from palm tree dried male inflorescence; hereafter denoted simply as ash), Batch III-0.1% sodium bicarbonate (NaHCO\(_3\)), Batch IV-1% palm sugar (hereafter denoted simply as sugar), Batch V-distilled water. The soaking solutions were discarded after the soaking period. The non-edible hard seed coats of *P. roxburghii* were removed and kernels alone were used for autoclaving. This soaked whole seeds of *A. auriculiformis* and kernels of *P. roxburghii* were autoclaved with their appropriate fresh soaking solution in 1:5 and 1:3 ratio (w/v) respectively at 121 °C for 20 and 15 min respectively.
3.2.2.2 Autoclaving without soaking

The VI\textsuperscript{th} batch of \textit{A. auriculiformis} seeds were subjected to autoclaving (without any of the soaking regimens) with distilled water in 1:10 ratio (seed:water, w/v) for 25 min. Hereafter these seeds were denoted as directly autoclaved seeds. This is not applied for \textit{P. roxburghii} since they have non-edible, hard seed coat.

3.2.2.3 Fermentation

Fermentation has been carried out only for \textit{P. roxburghii} seeds (VI\textsuperscript{th} Batch) since it is the major processing method for the seeds of \textit{Parkia} spp. One set of water soaked kernels were ground into coarse slurry with 1:3 ratio of water and autoclaved as said above. The slurry was kept for natural fermentation (solid state) at room temperature for 36 h. Fermentation process was terminated by exposing the slurry to aerobic conditions and allowing it to dry at 45±2 °C.

3.2.2.4 Dry heating

The VII\textsuperscript{th} batch both the legumes were mixed with acid washed sand particles (to ensure uniform heat distribution) and dry heated in a hot air oven at 105 °C/5 min and 130 °C/20 min for \textit{A. auriculiformis} and \textit{P. roxburghii} respectively. The seed coats of \textit{P. roxburghii} have been removed to obtain dry heated kernels after attaining the room temperature.

3.2.2.5 Handling of raw and processed seeds

All of the autoclaved seeds and kernels (II\textsuperscript{nd} - V\textsuperscript{th} batch of both the legumes and VI\textsuperscript{th} batch of \textit{A. auriculiformis}) were drained from the excess autoclaving solutions and allowed to dry at 45±2 °C. The processed, dried seeds and kernels along with raw ones were ground into fine powder using laboratory blender, followed by ball mill MM400 (Retsch, Germany) and stored in air tight polythene bags at 4 °C until further analysis. Similarly, roasted seeds and kernels were also ground and stored after attaining the ambient temperature.

3.2.3 Proximate composition

The moisture content of raw and processed samples were determined using Moisture Analyzer MA35 (Sartorius AG, Germany) at 105 °C. Micro-Kjeldahl method was employed to determine the total nitrogen and a nitrogen-protein conversion factor 6.25 is used for
crude protein (N×6.25) determination. Crude lipid (soxhlet extraction), crude fibre and ash contents (gravimetric) were also determined based on the methods outlined in Association of Official Analytical Chemists (AOAC) (1990). The carbohydrate (also called Nitrogen Free Extractives (NFE)) content was estimated by the percentile difference from all the other constituents. The proximate composition was expressed as g/100 g DM. The gross energy (kJ) was determined by multiplying the percentage of crude protein, crude lipid and NFE by 16.7, 37.7 and 16.7 respectively (Siddhuraju et al., 1996a).

3.3 Analysis of anti-nutritional factors

Raw and processed seed and kernel flours of A. auriculiformis and P. roxburghii have been analyzed for the following anti-nutritional factors.

3.3.1 Total phenolics and tannins

Total phenolic content (TPC) have been estimated by Folin-Ciocalteu method according to Makkar et al. (2007). In brief, 250 mg of seed flour was extracted with 10 mL of 70% acetone at room temperature in an ultrasonic water bath at 80 W for 30 min. The contents were centrifuged at 3000 ×g for 10 min at 4 °C and the supernatant was collected. Aliquot of the extracts to the final volume 500 μL with distilled water was mixed with 250 μL of Folin-Ciocalteu reagent (1N). 1.25 mL of 20% sodium carbonate solution was added. The contents were mixed well and incubated for 40 min under dark conditions. Absorbance was recorded at 725 nm against the blank. TPC was calculated as tannic acid equivalents (TAE) from the tannic acid calibration curve (2-10 μg tannic acid) and the results were expressed as g TAE/100 g DM.

Tannins in the extracts were estimated after their treatment with polyvinyl polypyrrolidone (PVPP) as per Makkar et al. (2007). 100 mg PVPP in 1 mL of distilled water was mixed with 1 mL of extract. The contents were vortexed and kept at 4 °C for 15 min. Then the samples were centrifuged at 3000 ×g for 10 min and the supernatant was collected. The supernatant contains simple phenolics (tannins have been bounded with the PVPP) have been estimated as mentioned above. The content of nontannin phenolics was calculated as g TAE/100 g DM. From the above results, the tannin content of the samples were calculated as,

\[
\text{Tannin (%)} = \text{Total phenolics (%)} - \text{Nontannin phenolics (%)}
\]
3.3.2 Condensed tannins

Condensed tannins also called as proanthocyanidins was estimated according to the method of Porter et al. (1986), outlined by Makkar et al. (2007). 0.5 mL of the above prepared extracts in glass tubes was mixed with 3 mL of butanol-HCl (95:5, v/v) reagent and 0.1 mL of the ferric ammonium sulphate reagent (2% ferric ammonium sulphate in 2N HCl). The contents in the tubes were mixed well, covered with marbles and kept in a heating block at 100 °C for 60 min. The tubes were allowed to cool and the absorbance was recorded at 550 nm and suitable blank was subtracted, which is usually the absorbance of unheated mixture. The condensed tannins were calculated as g leucocyanidin equivalents (LE) by the following formula,

\[
\text{Condensed tannins/100 g DM} = \frac{(\text{OD value at 550 nm} \times 78.26 \times \text{Dilution factor})}{(\% \text{ DM})}.
\]

3.3.3 Phytic acid

Phytic acid content of legume seeds was estimated by the method of Vaintraub and Lapteva (1988), outlined by Makkar et al. (2007). The extract for phytic acid estimation was obtained by mixing 5 g of seed flour with 100 mL of 3.5% HCl followed by stirring at room temperature for 1 h. The contents were centrifuged at 3000 × g for 10 min at room temperature and the supernatant was collected. 1 mL of the supernatant was diluted to 25 mL with distilled water. 3 mL of the above-diluted extract was mixed with 1 mL of Wade reagent (30 mg of FeCl₃.6H₂O and 300 mg of sulfosalicylic acid were dissolved in 70 mL of distilled water and made up to 100 mL with distilled water). The contents were vortexed and centrifuged at 3000 × g for 10 min. The absorbance of the supernatant was measured at 500 nm. Sodium phytate (100 g sodium phytate=59.9 g phytic acid) is used as reference. The content of phytic acid was expressed as g/100 g DM calculated from the phytate standard graph (32-160 µg phytic acid).

3.3.4 Saponins

Total saponin content of legume seeds was determined by the method of Hiai et al. (1976), outlined by Makkar et al. (2007). 10 g of defatted seed flour was mixed with 100 mL of 80% methanol and kept in a magnetic stirrer overnight at room temperature. The contents were centrifuged at 3000 × g for 10 min and the supernatant was collected. The extraction was again repeated with 80% methanol and both the supernatants were pooled.
together. Methanol from the extract was evaporated and the aqueous phase was centrifuged at 3000 \( \times g \) for 10 min to remove the water insoluble materials. Extract with concentrated saponins in the aqueous solution was mixed with equal volume of n-butanol. The solvent n-butanol was evaporated under vacuum and the dried residues were collected and the percent recovery of saponins was calculated. Known amount of saponin residues dissolved in 80\% aqueous methanol was used for estimation of total saponins. Aliquots of extract made up to the final volume of 0.25 mL was mixed with 0.25 mL of vanillin reagent (8\% vanillin in absolute ethanol). 2.5 mL of 72\% (v/v) sulphuric acid was added slowly in the inner side of the wall. The solution was mixed well and the tubes were transferred to a water bath at 60 °C for 10 min. The tubes were cooled in ice-cold water for 5 min at the end of incubation and the absorbance was recorded at 544 nm against the blank. The total saponin content was determined using diosgenin calibration graph (20-100 µg diosgenin) and the result was expressed as g diosgenin equivalents (DE)/100 g DM.

### 3.3.5 Trypsin inhibitor activity

Trypsin inhibitor activity was measured according to Kakade et al. (1969), modified by Smith et al. (1980) and outlined by Makkar et al. (2007). The extract was prepared by mixing 1 g of defatted seed flour with 50 mL of 0.01M NaOH for 3 h at room temperature on a magnetic stirrer at low speed. The extract was centrifuged at 3000 \( \times g \) for 10 min. The extraction was repeated for the samples which have the pH less than 8.4 at the end of 3 h incubation (The pH of the suspension is usually 9.5 to 9.8), with stronger NaOH solution. This suspension should be diluted to the point where 1 mL of the diluted suspension produces trypsin inhibition of 40 to 60\%.

Four set of tubes marked as a, b, c and d has been taken with the following solutions, a (Reagent blank) - 2 mL of distilled water; b (Enzymed standard: 20 µg trypsin/mL of 0.001M HCl) - 2 mL of trypsin solution and 2 mL of distilled water); c (Sample blank) - 1 mL of extract and 1 mL of distilled water; d (Sample) - 1 mL of extract, 1 mL of distilled water and 2 mL of trypsin solution. All the tubes were mixed well and 5 mL of benzyl-DL-arginine-para-nitroanilide (BAPNA) solution (40 mg BAPNA dissolved in 1 mL of DMSO was made up to 100 mL with Tris buffer (0.05M, pH 8.2 containing 0.02M CaCl\(_2\); pre-warmed at 37 °C) was added, mixed well and incubated at 37 °C for 10 min. At the end
of incubation, 1 mL of 30% acetic acid was added to arrest the reaction. 2 mL of trypsin solution was added to reagent blank (a) and sample blank (c) tubes. The contents were centrifuged at 3000 ×g for 10 min at room temperature. Absorbance of the supernatant was read at 410 nm. Percentage inhibition was calculated as,

\[ \frac{AI}{(Ab - Aa)} \]

Where, \( AI = (Ab - Aa) - (Ad - Ac) \). Lowercase letters a, b, c and d refers the tubes. From this, trypsin inhibitor activity was calculated as mg pure trypsin inhibited/g sample.

\[ TIA = \frac{(2.632 \times D \times AI)}{S} \]

Where, \( D \) is the dilution factor (the factor by which the original plant extract (1 g in 50 mL) was diluted so as to obtain an inhibition between 40-60% by 1 mL of the diluted extract), and \( S \) is the sample weight (g).

3.3.6 Chymotrypsin inhibitor activity

The chymotrypsin inhibitor activity of raw and processed legume seed flours was determined by the method of Kakade et al. (1970), outlined by Makkar et al. (2007) based on spectrophotometric determination of breakdown products of casein at 280 nm. The extract for the analysis was prepared by mixing 1 g of defatted flour with 10 mL of distilled water. The pH of the solution was adjusted to 7.6 and blended on a magnetic stirrer for 1 h at room temperature. The mixture was centrifuged at 3000 ×g for 10 min and the supernatant obtained was used for the analysis.

Aliquots of the extracts (in triplicates/extract) were made to the final volume of 1 mL with borate buffer (0.1M, pH 7.6) followed by the addition of 1 mL of chymotrypsin (40 µg chymotrypsin/mL of 0.001M HCl containing 0.08M CaCl₂·2H₂O). 6 mL of TCA reagent (18 g TCA + 18 g anhydrous sodium acetate + 20 mL acetic acid to the final volume of 1000 mL) added to one of the triplicate which served as blank for the other replicates. 2 mL of pre-warmed (37 °C) casein solution (1 g casein suspended in 80 mL of borate buffer 0.1M, pH 7.6 was dissolved by heating in a steam bath for 15 min. The solution was allowed to cool and the pH was adjusted to 7.6. The final volume was made up to 100 mL with the same borate buffer) was added to all the tubes and incubated for 10 min. The reaction was arrested by the addition of 6 mL of TCA to other two replicates. The reaction mixture was kept at room temperature for 30 min, filtered and the absorbance of the filtrate was
measured at 275 nm against the respective blank. The results were expressed as chymotrypsin units inhibited (CUI)/g sample using standard chymotrypsin calibration curve (2-8 µg chymotrypsin) performed under the same experimental conditions.

3.3.7 α-Amylase inhibitor activity

α-Amylase inhibitor activity was determined according to Deshpande et al. (1982), outlined by Makkar et al. (2007) by estimating the reducing sugars liberated by the enzyme using dinitrosalicylic acid (DNSA) reagent (Sumner, 1924). The enzyme extract of legume seeds was prepared as follows; 1g of seed flour was extracted with 10 mL of distilled water for 12 h at 4 °C using a magnetic stirrer. The contents were centrifuged at 5000 ×g for 20 min and the supernatant was collected.

α-Amylase enzyme activity was determined before determining the inhibitor activity. All reagents prepared were pre-warmed for 15 min at 37 °C in a water bath. The first set of tubes with 0.5 mL of 1% starch solution in 0.02M phosphate buffer (pH 7.0) was added with 0.25 mL of the phosphate buffer (0.2M, pH 7.0) and 0.25 mL of α-amylase enzyme solution (30 µg α-amylase/mL of 0.2M phosphate buffer (pH 7.0) containing 0.006M NaCl). The second set of tubes containing phosphate buffer in place of the enzyme solution serves as blank. The third set of test tubes containing 0.5 mL of starch solution, 2 mL of DNSA (1 g of 3,5-dinitrosalicylic acid in 20 mL of 2N NaOH + 50 mL of distilled water + 30 g of Rochelle salt. The contents were dissolved and made up to 100 mL with distilled water), 0.25 mL of the phosphate buffer and 0.25 mL of α-amylase enzyme solution has been set as zero time control. The tubes were incubated at 37 °C for 3 min. 2 mL of DNSA reagent was added to the first and second set of tubes at the end of the incubation to stop the reaction. All the tubes were kept in boiling water bath for 10 min. After cooling the tubes, 10 mL of distilled water was added, mixed thoroughly and the absorbance was taken at 540 nm against the blank. Liberated reducing sugars are expressed as maltose equivalents using the calibration curve (0.5-5 µmol maltose). One unit of α-amylase enzyme activity is defined as, the amount which liberates 1 µmol of reducing groups (calculated as maltose)/min from soluble starch at 37 °C, pH 7.0, under the specified experimental conditions.

In order to determine the inhibitory activity, 0.25 mL of extract from seed flours was incubated with 0.25 mL of enzyme solution for 15 min at 37 °C. To this mixture, 0.5 mL of
1% starch solution was added and the assay was repeated as described above. The sample blank was prepared with the addition of 0.25 mL of \( \alpha \)-amylase enzyme solution at the end of 3 min incubation after the addition of DNSA reagent. One unit of \( \alpha \)-amylase activity inhibited is defined as one \( \alpha \)-amylase inhibitory unit (AIU) and the result was expressed as \( \alpha \)-amylase inhibitor units/g sample.

### 3.3.8 Lectins

Lectins also called phytohemagglutinins in legume flours were determined by the method of Gordon and Marquardt (1974), outlined by Makkar et al. (2007). The lectin extract was prepared by mixing 1 g of defatted seed and kernel flour with 20 mL of phosphate buffered saline (PBS) followed by stirring at 4 °C for 16 h. The contents were centrifuged at 4000 \( \times g \) for 20 min at 4 °C and supernatant collected was used for detecting the agglutinating activity. Blood from healthy subjects of rat, cow, goat and human (A, B and O) was collected in a tube containing ethylene diamine tetra acetic acid (EDTA)-disodium salt as an anti-coagulant. Blood was centrifuged immediately at 1500 \( \times g \) for 5 min; the plasma and buffy coat was discarded. The packed erythrocytes/red blood corpuscles (RBC’s) were washed four times with PBS in the ratio of 1:5 (v/v). 4 mL of washed RBC’s was diluted with 95 mL of PBS and 1 mL of trypsin solution (10 mg/mL PBS) and incubated at 37 °C for 1 h. The trypsinized RBC’s were washed three times with PBS. From the trypsinized RBC’s, 2 (cow and goat RBC’s) and 3% (rat and human RBC’s) suspension was prepared for the determination of agglutinating activity (Tan et al., 1983; Makkar et al., 1997).

Two fold serial dilution of the extract was carried out with the PBS in the wells of U-bottom microtiter plate and mixed with equal volume of trypsinized erythrocyte suspensions (final volume 0.1 mL). The plates were incubated at room temperature for 2 h and the presence of agglutination was analyzed. A uniform effacement of the bottom of the well by RBC’s indicates positive agglutination and a circular clump of RBC’s surrounded by a concentric clear zone indicates absence of agglutination. Hemagglutination activity is defined as the inverse of the amount of material/mL in the last dilution giving positive agglutination. The result was expressed as hemagglutinating units (HU)/g protein.
3.4 *In vitro* protein and *in vitro* starch digestibility

The *in vitro* protein digestibility (IVPD) of raw and processed samples was determined by multienzyme technique (Satterlee, 1979). Legume seed flours containing 62.5 mg protein was suspended in 10 mL of distilled water and the pH was adjusted to 8.0. The contents were stirred at 37 °C for 15 min. One mL of multienzyme solution was added to the protein suspension and again stirred at 37 °C for 10 min. The enzyme solution consists of 1.6 mg trypsin (14000 U/mg), 3.1 mg chymotrypsin (48 U/mg) and 1.3 mg peptidase (102 U/g) /mL, prepared in ice bath and the pH was adjusted to 8.0. After 10 min of incubation, 1 mL protease solution (7.5 mg bacterial protease type XIV from *Streptomyces griseus*/mL distilled water) was added and the reaction mixture was transferred to 55 °C water bath for 9 min. At the end of incubation, the tubes were transferred to 37 °C water bath for a minute. The pH of the reaction mixture was recorded and the protein digestibility was calculated by using the following regression equation,

\[ Y = 234.84 - 22.56X \]

Where, \( Y \)=protein digestibility % and \( X \)=pH of the solution after 20 min digestion with 4-enzyme solution.

*In vitro* starch digestibility (IVSD) was determined by the method of Goni *et al.* (1997). Before determining digestibility rate, total starch content was estimated as follows; 50 mg of sample was dispersed with 6 mL of 2M KOH and kept at constant shaking for 30 min. This was followed by the addition of 80 µL amyloglucosidase to hydrolyze the solubilized starch for 45 min at 60 °C. The contents were centrifuged at 3000 ×g for 15 min, filtered and the glucose content was estimated using glucose oxidase/peroxidase reagent (Sigma Chemicals and Co, St. Louis, MO, USA.) according to the supplier’s specifications. The total starch was calculated from the glucose content estimated by multiplying glucose content × 0.9. The total starch was corrected for free glucose content and glucose moiety of sucrose as follows; samples dispersed in 2M KOH were incubated with invertase enzyme solution for 30 min at 37 °C. The contents were centrifuged at 3000 ×g for 10 min. One mL of the supernatant was mixed with 2 mL of 96% ethanol and centrifuged at 3000 ×g for 10 min. Glucose content of the supernatant was estimated by glucose oxidase/peroxidase reagent. The free glucose and glucose moiety of sucrose estimated, was subtracted with total starch content to get the corrected total starch content from the interfering glucose moieties.
50 mg of samples were mixed with 10 mL of HCl-KCl buffer (pH 1.5) and 0.2 mL of pepsin solution (1 g/10 mL HCl-KCl buffer, pH 1.5). The reaction mixture was incubated at 40 °C for 1 h in a shaking water bath. At the end of incubation, the reaction mixture was made up to 25 mL with Tris-maleate buffer pH 6.9 followed by the addition of 5 mL of α-amylase solution (2.6 IU) in Tris-maleate buffer pH 6.9. This was incubated for 3 h at 37 °C in a shaking water bath. 1 mL of aliquot from the mixture was taken and incubated at 100 °C for 5 min under vigorous shaking conditions to inactivate the enzyme. Then 3 mL of 0.4M sodium acetate buffer pH 4.75 was added to the aliquots and 60 µL of amyloglucosidase was added and incubated for 45 min at 60 °C in a shaking water bath to hydrolyze the digested starch into glucose. The volume was adjusted to 10-100 mL with distilled water. Triplicate aliquots of 0.5 mL were incubated with a glucose oxidase-peroxidase reagent. The glucose was converted into starch by multiplying with 0.9. Starch digestibility percentage was calculated as % starch hydrolyzed from the total starch content of the sample.

3.5 Amino acid analysis

Amino acid contents of the raw and processed seed and kernel flours were determined by high performance liquid chromatography (HPLC). The samples were hydrolyzed by 6 N HCl at 110 °C for 24 h under vacuum (AOAC, 1995). The amino acids were analyzed by HPLC equipped with a fluorescence detector (Agilent, Santa Clara, CA, USA). The analytical column was a reversed phase C18 column (Nova-Pak, 150×3.9 mm i.d. and 4 µm particle size, Waters, Milford, MA, USA). Amino acid standards were used for peak identification and quantification. 2.5 mM L-α-amino-n-butyric acid was used as an internal standard.

3.6 Fatty acid analysis

The seed and kernel oil were extracted using petroleum ether (40-60 °C) in the ratio of 1:20 (w/v) for 8 h at room temperature. After centrifugation, the solvent was removed under a nitrogen atmosphere. Methyl esters were prepared from the total lipids by the method of AOAC (1990). These fatty acid methyl esters were analyzed by gas chromatography (GC-14A, Shimadzu, Japan) using an instrument equipped with a flame ionisation detector (FID). The column temperature was set as gradient ranged from 160 to 225 °C and nitrogen, at a flow rate of 1 mL/min, was used as a carrier gas. A standard fatty acid methyl ester mixture
was run and retention times were used in identifying the sample peaks. Fatty acid contents were estimated from the peak area of standard and sample methyl esters.

### 3.7 Mineral analysis

One gram of raw and processed seed flours were digested with a mixture of concentrated nitric acid, sulfuric acid and 60% perchloric acid (9:2:1, v/v). After cooling the digest was diluted with 50 mL of distilled water, filtered with Whatman No. 1 filter paper and the filtrate was made up to 100 mL with distilled water. The minerals sodium (Na), potassium (K), calcium (Ca) were determined by flame photometer (Pearson, 1976). Total phosphorous (P) was estimated by the ascorbic acid method after acid digestion and neutralization by phenolphthalein indicator and combined reagent with KH$_2$PO$_4$ as standard (APHA, 1995). Remaining minerals (magnesium (Mg), iron (Fe), copper (Cu), zinc (Zn) and manganese (Mn)) were estimated by atomic absorption spectrophotometer (Perkin-Elmer, Model 5000). Standard calibration curve of known concentrations were made from pure metal/metal oxide.

### 3.8 Effect of processing methods on antioxidant power

#### 3.8.1 Solvent extraction

The raw and processed seed and kernel flours were defatted with petroleum ether. The air-dried residues were extracted with 80% methanol in the ratio of 1:5 (w/v) by maceration. The extracts obtained were dried at 40 °C until getting a constant weight.

#### 3.8.2 Determination of total phenolic content

The TPC of extracts were determined according to Folin-Ciocalteu method at 725 nm described by Makkar et al. (2007). Aliquots of the extracts to final volume of 1 mL, was added with 0.5 mL of Folin-Ciocalteu reagent (1N) and 2.5 mL of sodium carbonate solution (20%). Soon after vortexing the reaction mixture, the tubes were placed in the dark for 40 min and the absorbance was recorded at 725 nm against the blank. TPC of extracts were expressed as mg gallic acid equivalents (GAE)/g extract calculated with gallic acid calibration graph (2-10 µg gallic acid).
3.8.3 Ferric reducing antioxidant power (FRAP) assay

FRAP was done by the method of Benzie and Strain (1996). An aliquot of 30 μL extracts were mixed with 90 μL of water and 900 μL of FRAP reagent (2.5 mL of 20 mmol/L 2,4,6 [Tri(2-pyridyl)-s-triazine] (TPTZ) in 40 mmol/L HCl + 2.5 mL of 20 mmol/L ferric chloride + 25 mL of 0.3 mol/L acetate buffer (pH 3.6)) and incubated at 37 °C for 30 min. Absorbance was recorded at 593 nm and the reducing power was expressed as mmol Fe (II)/g extract using the calibration graph of ferrous sulphate (0.4-2.0 mmol/L ferrous sulphate). The positive standards used for comparison were BHA and rutin.

3.8.4 Iron chelating activity

The ability of the extracts and standards to chelate ferrous ion was determined by the method of Dinis et al. (1994). 0.1 mL of sample, 0.6 mL of distilled water and 0.1 mL of 2 mmol/L FeCl₂ were mixed well, and incubated for 30 sec. 0.2 mL of 5 mmol/L ferrozine was added to the above mixture, incubated for 10 min at room temperature and the absorbance was recorded at 562 nm. Results were expressed as mg EDTA equivalents/g extract using EDTA calibration graph (0.5-1.5 µg EDTA) and compared with the positive control BHA.

3.8.5 Free radical scavenging activity on 2,2′-diphenyl-1-picryl-hydrazyl (DPPH˙)

The radical scavenging activity of extracts was measured using DPPH radical by the method of Brand-Williams et al. (1995) with some modifications. Extract/standard of 0.1 mL prepared in methanol was mixed with 3.9 mL of DPPH˙ (6×10⁻⁵ mol/L methanol) and incubated in dark for 30 min. Absorbance was read at 515 nm and the results were expressed mmol trolox equivalents (TE)/g extract using trolox calibration graph (0.5-2.5 mmol/L trolox). Positive standards of BHA and rutin were used for comparison.

3.8.6 Free radical scavenging activity on 2,2-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt (ABTS⁺⁺)

The ABTScationic radical (ABTS⁺⁺) decolourization assay was done by the method of Re et al. (1999). ABTS⁺⁺ was generated by the interaction of potassium persulphate (2.45 mM) with ABTS (7 mM). The reaction mixture was incubated in dark at room temperature for 12-16 h and diluted with ethanol to give an absorbance of 0.70±0.02 at 734 nm. 1.0 mL of this ABTS⁺⁺ solution was mixed with 10 μL of extracts/standards and
incubated at 30 °C for 30 min. The absorbance was measured at 734 nm and the results were expressed as mmol trolox equivalents (TE)/g extract using trolox calibration graph (0.25-1.5 mmol/L trolox) and compared with the positive controls BHA and rutin.

3.8.7  **Determination of scavenging activity on biologically relevant radicals**

3.8.7.1 **Hydroxyl radical scavenging activity**

Hydroxyl radical (‘OH) scavenging activity of extracts/standards was measured using ascorbic acid-iron-EDTA model (Klein et al., 1991). Extracts/standards were mixed with 1 mL of iron-EDTA solution (0.13% ferrous ammonium sulphate in 0.26% EDTA), 0.5 mL of 0.018% EDTA and 1 mL of DMSO solution (0.85%, v/v in phosphate buffered saline 0.1M, pH 7.4). The reaction was initiated by the addition of 0.5 mL of 0.22% ascorbic acid and incubated at 80-90 ºC in a water bath for 15 min. After the incubation, the reaction was terminated by the addition of 1 mL of ice cold TCA (17.5, w/v). 3 mL of Nash reagent (75 g ammonium acetate + 3 mL glacial acetic acid + 2 mL acetyl acetone/L of reagent) was added to the above mixture and allowed to stand at room temperature for 15 min for color development. Absorbance values were recorded at 412 nm. The % hydroxyl radical scavenging activity was calculated using the following formula,

\[
\% \text{ scavenging activity} = 1 - \left( \frac{\text{Difference in absorbance of sample}}{\text{Difference in absorbance of control}} \right) \times 100
\]

The activity was compared with the positive standard catechin (250 μg).

3.8.7.2 **Superoxide anion radical scavenging activity**

The superoxide anion radical (O$_2^-$) scavenging capacity of extracts was determined by the method of Martinez et al. (2001) for the determination of superoxide dismutase with some modifications made by Dasgupta and De (2004) in the riboflavin-light-nitrobluetetrazolium system (Beauchamp and Fridovich, 1971). Each 3 mL of reaction mixture consists of 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, 75 μM NBT and 1 mL extract/standard was kept for 10 min of illumination under 20W fluorescent lamps. The production of blue formazan was monitored and recorded at 560 nm. The degree of superoxide anion radical scavenging activity was calculated as follows,

\[
\% \text{ scavenging activity} = \left( \frac{A_c - A_s}{A_c} \right) \times 100
\]

Where, $A_c$ - Absorbance of control; $A_s$ - Absorbance of sample.
3.8.7.3 Nitric oxide scavenging activity assay

Nitric oxide (NO\textsuperscript{•}) scavenging activity was done by the method of Marcocci et al. (1994). Extracts/standards (at a concentration of 500 µg) were mixed with sodium nitroprusside (5 mM final concentration) in phosphate buffered saline pH 7.4 to the final volume of 1 mL and incubated at 25 °C for 150 min. After incubation, the reaction mixture was mixed with Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% ortho phosphoric acid). The absorbance was measured at 540 nm. The degree of NO\textsuperscript{•} scavenging activity was calculated as follows,

\[
\% \text{ scavenging activity} = \frac{(A_c - A_s)}{A_c} \times 100
\]

Where, \(A_c\) - Absorbance of control; \(A_s\) - Absorbance of sample. The scavenging activity was compared with the positive standards (150 µg) BHA, rutin and trolox.

3.8.8 β-Carotene bleaching assay

Peroxidation inhibition (PI) on lipid molecules was measured using β-carotene-linoleic acid double system by monitoring the β-carotene bleaching (Taga et al., 1984). Stock solution of β-carotene-linoleic acid mixture was prepared as follows: 2 mg of β-carotene was dissolved in 1 mL of chloroform along with 40 mg of linoleic acid and 400 mg of Tween 40. Tween 40 is added for its emulsifying property since β-carotene is not water soluble. Chloroform is allowed to evaporate. To this, 100 mL of distilled water was added and the mixture was vigorously mixed until it is uniformly distributed. 4.8 mL of this reaction mixture was mixed with 0.2 mL of extracts/standards and the absorbance was measured at 470 nm immediately against blank consists of reaction mixture without β-carotene. 4.8 mL of this reaction mixture was mixed with 0.2 mL of distilled water serves as control. All the tubes were kept in a water bath at 50 °C and the absorbance was measured at 120 min. PI was calculated as,

\[
\text{PI\%} = 1 - \frac{(A_{E0} - A_{E120})/(A_{C0} - A_{C120})}{A_{C0}} \times 100
\]

Where \(A_{E0}\) and \(A_{C0}\) are the absorbance values measured at zero time of the incubation of extract and control tubes respectively. \(A_{E120}\) and \(A_{C120}\) are the absorbance values measured in the extract and control at 120 min respectively.

3.8.9 Oxidative hemolysis inhibition assay

Protective action of raw and processed legume extracts against hemolysis is followed by Valente et al. (2011). Blood (10 mL) was collected from healthy human volunteers by
venipuncture in a citrated tube. It was centrifuged immediately at 1500 rpm for 10 min at 4 °C and washed three times with phosphate buffered saline (PBS 0.02 M, pH 7.4) to get RBC’s. It is re-suspended to 2% using the same buffer. To study the protective effects of the extracts/standards against 2,2’-azobis (2-amidinopropane) dihydrochloride (AAPH) - induced hemolysis, RBC’s were pre-incubated with the extracts/standards at 37 °C for 30 min, followed by incubation with and without AAPH (dissolved in PBS; final concentration 50 mM). This reaction mixture was shaken gently while being incubated for 4 h at 37°C. A negative control (RBC’s in PBS), as well as extract controls (RBC’s in PBS with extract) were used for comparison. The extent of hemolysis was determined spectrophotometrically by the procedure of Ko et al. (1997). Briefly, the reaction mixture was centrifuged at 4000 rpm for 10 min to separate the RBC’s at the end of incubation and the data were calculated for antihemolytic activity.

\[
\% \text{ antihemolytic activity} = \frac{(A_c - A_s)}{A_c} \times 100
\]

Where, \(A_c\) - Absorbance of control; \(A_s\) - Absorbance of sample.

The following positive standards at a concentration of 50 μg were used for comparison: BHA, rutin and trolox.

3.8.10 DNA protection assay

The DNA protection assay was performed using pBR322 plasmid DNA by the method of Hiramoto et al. (2003) with some modifications. The reaction mixture (20 μL) consists of 2 μL pBR322 (200 ng), 2 μL of 50 mM AAPH (final concentration 5 mM) and 16 μL of extracts dissolved in buffer (50 μg) is incubated at room temperature for 10 min. Then the contents were run on 1% agarose gel under 50V for 1 h. The measurement of DNA damage was initially visualized by UV-transilluminator ECX (Vilber lourmat, France) and documented by Geldoc lab image IDL 320 (Medicare Scientific, India).

3.9 Fish feeding trial

Potency of raw and best processed seeds and kernels of A. auriculiformis (ash soaked and autoclaved seeds) and P. roxburghii (fermented kernels) as a partial substitution of fish meal on the growth performance of three major Indian carps, catla (Catla catla), rohu (Labeo rohita) and mrigal (Cirrhinus mrigala) have been investigated in a 60 days feeding trial.
3.9.1 Experimental diets formulation

The control and experimental diets have been formulated according to Siddhuraju and Becker (2001a) and Nandeesha et al. (2001). The sterilized fish meal is obtained from Sri RMP feeds, Palladam, Coimbatore, TN, India. The other ingredients such as groundnut oil cake and rice bran were obtained from local market. Prior to diet formulation, proximate composition of diet ingredients were analyzed (Table 3.2).

The entire experimental and control diets were formulated at nearly isonitrogenous (40 g/100 g feed) and isocalorific (1800 kJ/100 g feed). The CTR acted as control diet (40% crude protein) with protein source from fish meal and groundnut oil cake (containing no added ingredients of either A. auriculiformis or P. roxburghii seed meal). Eight experimental diets were formulated (Table 3.3) in order to evaluate the efficiency of raw and processed seed meals of A. auriculiformis and P. roxburghii as a fish meal substitute in fish feeds. The experimental diets were designated as AR10%, AR20% (formulated with A. auriculiformis raw seed meal), AA10%, AA20% (formulated with A. auriculiformis ash soaked and autoclaved seed meal), PR10%, PR20% (formulated with P. roxburghii raw kernel meal), PF10%, PF20% (formulated with P. roxburghii fermented kernel meal). 10% and 20% indicates the inclusion level of crude protein from the test ingredients in the total protein content (40%) of the control diet. All the ingredients were thoroughly mixed with water into dough consistency and made into pellets through locally fabricated pelletizer to obtain 2.5 mm diameter pellets. The pelleted diets were shade dried followed by incubation at 40 °C to improve the durability and stored in plastic containers. Proximate composition of control and experimental diets were given in Table 3.4.

3.9.2 Feeding trial

The animals were obtained from Tamil Nadu Fisheries Corporation, Aliyar, Pollachi, TN, India. The feeding trial was conducted with three major Indian carps catla, rohu and mrigal reared separately for a period of 60 days. The experiments were carried out in glass tanks (60×50×50 cm) with the total of 27 treatment groups (9 groups/fish species) with two replicates in each treatment in the natural photoperiod of 12L:12D. Water from upper Aliyar reservoir was used for rearing of fishes. Water quality parameters such as pH, dissolved oxygen and ammonia were monitored on weekly basis. Initial body weight of 6.2±0.2 g,
5.6±0.2 g and 5.3±0.3 g for catla, rohu and mrigal respectively (15 fish/replicate) has been used for the study. The fishes were allowed to acclimatize the experimental system for the period of 7 days before starting the experiment. At the beginning of the study 10 fish/fish species have been sampled and frozen at -20 °C for the analysis of initial body proximate composition. The fishes were fed with near satiation (5% of body weight), twice the day at 9.00 and 14.00 h. The pelleted feeds were crushed into crumbs before administering them to fish. Daily observations were made for the feeding behavior of all fish species and mortality was recorded. All the fish were sampled once a week to record the weight and feed quantity for the subsequent period was calculated. The cisterns were cleaned on daily basis and replaced with fresh water. At the end of the 60th day, the respective control and experimental fishes were sacrificed followed by homogenization and freeze drying of whole body of fish. They were analyzed for proximate composition.

3.9.3 Analytical methods

Proximate composition of feed ingredients, experimental diets and initial and final fish carcass have been analyzed by the standard methods of the AOAC (1990). The gross energy was determined by bomb calorimeter using benzoic acid as a standard.
Table 3.2 Proximate composition of individual feed ingredients of control and experimental diets (g/100 g)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AR</th>
<th>AA</th>
<th>PR</th>
<th>PF</th>
<th>Fish meal</th>
<th>Groundnut oil cake</th>
<th>Rice bran</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>8.1±0.3</td>
<td>7.7±0.1</td>
<td>4.2±0.6</td>
<td>4.5±0.7</td>
<td>9.8±0.9</td>
<td>5.3±1.2</td>
<td>4.3±0.5</td>
</tr>
<tr>
<td>Crude protein</td>
<td>28.1±0.3</td>
<td>32.2±0.3</td>
<td>28.4±0.9</td>
<td>38.5±1.9</td>
<td>62.9±1.6</td>
<td>40.2±1.3</td>
<td>10.4±0.6</td>
</tr>
<tr>
<td>Lipid</td>
<td>13.7±1.4</td>
<td>12.0±0.7</td>
<td>21.7±0.4</td>
<td>30.9±1.2</td>
<td>11.5±0.9</td>
<td>6.2±1.1</td>
<td>5.3±0.5</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>7.4±0.2</td>
<td>8.6±0.8</td>
<td>5.8±0.4</td>
<td>3.2±0.9</td>
<td>0.8±0.4</td>
<td>5.7±1.0</td>
<td>8.5±0.9</td>
</tr>
<tr>
<td>Ash</td>
<td>3.6±0.1</td>
<td>3.7±0.3</td>
<td>5.1±0.3</td>
<td>4.5±0.3</td>
<td>12.5±0.6</td>
<td>3.9±0.6</td>
<td>3.1±0.9</td>
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<tr>
<td>NFE</td>
<td>39.2</td>
<td>35.9</td>
<td>34.8</td>
<td>18.4</td>
<td>2.5</td>
<td>38.7</td>
<td>68.4</td>
</tr>
<tr>
<td>Gross energy (kJ/100 g)</td>
<td>1631.5</td>
<td>1588.0</td>
<td>1873.5</td>
<td>2115.2</td>
<td>1525.7</td>
<td>1551.4</td>
<td>1515.8</td>
</tr>
</tbody>
</table>

Values are mean±SD (n=3). AR - *A. auriculiformis* raw seed flour; AA - *A. auriculiformis* ash soaked and autoclaved seed flour; PR - *P. roxburghii* raw kernel flour; PF - *P. roxburghii* fermented kernel flour.

Table 3.3 Formulation of control and experimental diets

<table>
<thead>
<tr>
<th>Ingredients (g/100 g)</th>
<th>CTR</th>
<th>AR10%</th>
<th>AR20%</th>
<th>AA10%</th>
<th>AA20%</th>
<th>PR10%</th>
<th>PR20%</th>
<th>PF10%</th>
<th>PF20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal</td>
<td>45</td>
<td>41</td>
<td>37</td>
<td>42</td>
<td>38</td>
<td>41</td>
<td>37</td>
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<tr>
<td>Rice bran</td>
<td>25</td>
<td>19</td>
<td>13</td>
<td>19</td>
<td>14</td>
<td>13</td>
<td>19</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Groundnut oil cake</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
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<tr>
<td>Raw/processed legume seed/kernel flour</td>
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<td>10</td>
<td>20</td>
<td>9</td>
<td>18</td>
<td>10</td>
<td>20</td>
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<td>Soybean oil</td>
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<td>Vitamin mineral premix</td>
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<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total (g)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Values are mean±SD (n=3). CTR-Control diet; AR10% and AR20% - Diets formulated with *A. auriculiformis* raw seed meal; AA10% and AA20% - Diets formulated with *A. auriculiformis* ash soaked and autoclaved seed meal; PR10% and PR20% - Diets formulated with *P. roxburghii* raw kernel meal; PF10% and PF20% - Diets formulated with *P. roxburghii* fermented kernel meal. 10% and 20% indicates the substitution level (on % basis) of crude protein in total protein content (40%) of the control diet. † Vitamin mineral premix (Emix™ Plus) (quantity/2.5 kg); vitamin A - 5500000 IU; vitamin D3 - 1100000 IU; vitamin B2 - 2000 mg; vitamin E - 750 mg; vitamin K - 1000 mg; vitamin B6 - 1000 mg; vitamin B12 - 6 mg; calcium pantothenate - 2500 mg; niacinamide - 10 g; choline chloride - 150 g; manganese - 27000 mg; iodine - 1000 mg; iron - 7500 mg; zinc - 5000 mg; copper - 2000 mg; cobalt - 450 mg; calcium - 500 g; phosphorus - 300 g; L-lysine - 10 g; DL-methionine - 10 g; selenium - 50 ppm; *Lactobacillus* 120 million units and yeast culture 3000 crore units.
Table 3.4 Proximate composition of control and experimental diet formulations (g/100 g)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diets</th>
<th>CTR</th>
<th>AR10%</th>
<th>AR20%</th>
<th>AA10%</th>
<th>AA20%</th>
<th>PR10%</th>
<th>PR20%</th>
<th>PF10%</th>
<th>PF20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>5.8±0.3</td>
<td>6.3±0.1</td>
<td>6.4±0.3</td>
<td>6.5±0.1</td>
<td>6.6±0.3</td>
<td>6.8±0.3</td>
<td>6.6±0.2</td>
<td>6.8±0.1</td>
<td>6.3±0.3</td>
<td></td>
</tr>
<tr>
<td>Crude Protein</td>
<td>40.4±0.5</td>
<td>40.6±0.6</td>
<td>40.7±0.2</td>
<td>40.7±0.6</td>
<td>40.8±0.7</td>
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<td>39.8±0.3</td>
<td>41.2±0.3</td>
<td>40.9±0.5</td>
<td></td>
</tr>
<tr>
<td>Lipid</td>
<td>7.9±0.2</td>
<td>7.7±0.3</td>
<td>7.4±0.3</td>
<td>7.6±0.3</td>
<td>7.8±0.2</td>
<td>8.4±0.3</td>
<td>8.9±0.3</td>
<td>9.2±0.3</td>
<td>10.2±0.4</td>
<td></td>
</tr>
<tr>
<td>Ash</td>
<td>5.3±0.3</td>
<td>5.4±0.2</td>
<td>5.6±0.3</td>
<td>5.7±0.1</td>
<td>5.8±0.1</td>
<td>5.6±0.3</td>
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<td>5.6±0.2</td>
<td>5.6±0.3</td>
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</tr>
<tr>
<td>Crude fibre</td>
<td>6.3±0.2</td>
<td>6.4±0.2</td>
<td>6.6±0.1</td>
<td>6.6±0.1</td>
<td>6.5±0.3</td>
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</tr>
<tr>
<td>NFE</td>
<td>34.2</td>
<td>33.5</td>
<td>33.4</td>
<td>32.9</td>
<td>32.5</td>
<td>32.1</td>
<td>33.4</td>
<td>31.7</td>
<td>31.3</td>
<td></td>
</tr>
<tr>
<td>Energy (MJ/kg)</td>
<td>18.4</td>
<td>18.8</td>
<td>18.9</td>
<td>18.3</td>
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<td>18.4</td>
<td>18.9</td>
<td>19.1</td>
<td>19.8</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SD (n=3). CTR - Control diet; AR10% and AR20% - Diets formulated with A. auriculiformis raw seed meal; AA10% and AA20% - Diets formulated with A. auriculiformis ash soaked and autoclaved seed meal; PR10% and PR20% - Diets formulated with P. roxburghii raw kernel meal; PF10% and PF20% - Diets formulated with P. roxburghii fermented kernel meal. 10 and 20 indicates the substitution level (on % basis) of crude protein in total protein content (40%) of the control diet.
3.9.4 Analysis of growth parameters

Growth and nutrient utilization was monitored and analyzed as follows according to the method of (Castell and Tiews, 1980).

Body weight gain (%) = \( \frac{(Final \ body \ weight \ - \ Initial \ body \ weight) \times 100}{Initial \ body \ weight} \)

Feed intake = Dry feed fed (g)/fish/day

Feed conversion ratio = \( \frac{Dry \ feed \ fed}{Live \ body \ weight \ gain} \)

Specific growth rate = \( \frac{(In \ final \ body \ weight \ - \ In \ initial \ body \ weight) \times 100}{Number \ of \ trial \ days} \)

Protein efficiency ratio = \( \frac{Wet \ body \ weight \ gain}{Crude \ protein \ fed} \)

Apparent net protein utilization = \( \frac{(Final \ fish \ body \ protein \ - \ initial \ fish \ body \ protein) \times 100}{Crude \ protein \ intake} \)

3.10 Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) and the significant difference between mean values was determined by Duncan’s multiple range test (P <0.05) using the SPSS (Statistical Package for the Social Sciences) version 13.0. (SPSS Inc., Chicago, Illinois, USA). Values were expressed as mean of triplicate determinations±standard deviation (SD).

3.11 Evaluation of health promoting potentials of A. auriculiformis bark and empty pods

In order to evaluate the health promoting aspects, the bark and empty pods of A. auriculiformis were chosen for the study.

3.11.1 Determination of antioxidant power

The bark and pods were collected during the month of June 2010, from a tree growing at Bharathiar University, Coimbatore, TN, India. The pods were removed from the seeds and aril. Both were cleaned with tap water to wash out the sand particles and dried in an oven at 40 °C and ground into fine powder using laboratory blender (Remi Anupam Mixie Ltd, Mumbai, Maharashtra, India). The powdered samples were defatted
with petroleum ether. The dried residues were extracted with 70% acetone in the ratio of 1:5 (sample:solvent, w/v) by maceration. The bark and empty pod extracts were dried at 40 °C and the yield (%) was calculated.

Both the extracts were analyzed for the following in vitro antioxidant assays,

1. Determination of total phenolics, flavonoids and proanthocyanidins
2. Ferric reducing antioxidant power (FRAP) assay
3. Iron chelating activity
4. Free radical scavenging activity on synthetic radicals DPPH and ABTS
5. Free radical scavenging activity on biologically relevant radicals
6. Lipid peroxidation inhibition by β-carotene bleaching system
7. Oxidative hemolysis inhibition assay on human RBC’s
8. DNA protection assay on pBR322

The materials and methods for this profile have been reported in earlier part of this section except the estimation of flavonoids. Hence it is given here.

Total flavonoid content was measured according to the method of Zhishen et al. (1999). 1 mL of sample was added to 10 mL volumetric flask containing 4 mL of water. At the onset of the experiment, 0.3 mL of 5% NaNO₂ was added to the flask. After 5 min, 0.3 mL of 1% AlCl₃ was added. After 6 min, 2 mL of 1M NaOH was added to the mixture and mixed well. Immediately, the solution was diluted to the final volume of 10 mL with water, mixed thoroughly and the absorbance was measured at 510 nm against the blank. Total flavonoid content was calculated using rutin standard graph (20-100 µg rutin) and expressed as mg rutin equivalents (RE)/g extract.

3.11.2 Determination of inhibition activity against key enzymes relevant to hyperglycemia

The extracts were additionally examined for their inhibiting potential against the key enzymes (α-amylase and α-glucosidase) relevant of hyperglycemia.

3.11.2.1 α-Amylase inhibitor activity

The bark and empty pod extracts were mixed with 100 µL of 0.02M sodium phosphate buffer (pH 6.9) and 100 µL of α-amylase solution (4.5 units/mL/min) and
pre-incubated at 25 °C for 10 min. Then, 100 µL of 1% starch solution was added and incubated at 25 °C for 30 min and the reaction was stopped by the addition of 1.0 mL of DNSA reagent. The test tubes were incubated in a boiling water bath for 5 min and then allowed to cool. The reaction mixture was then diluted to 10-fold times with distilled water and the absorbance was measured at 540 nm. The readings were compared with the control (extract was replaced by buffer) and α-amylase inhibition activity (%) was calculated (Worthington, 1993).

3.11.2.2 α-Glucosidase inhibitor activity

The bark and empty pod extracts were mixed with 100 µL of 0.1M phosphate buffer (pH 6.9) and 100 µL α-glucosidase solution (1 unit/mL/min) and incubated at 25 °C for 5 min. After the pre-incubation, 100 µL of p-nitrophenyl-α-D-glucopyranoside (5 mM) solution was added and the reaction mixture was incubated at 25 °C for 10 min. After the incubation, the absorbance was recorded at 405 nm and α-glucosidase inhibition (%) was calculated (Worthington, 1993).

3.12 In vivo studies

The identified health promoting potentials of bark and empty pods of A. auriculiformis in vitro were extended for the activity demonstration in vivo via hepatoprotective activity against paracetamol intoxicated liver injury and anti-diabetic activity against alloxan induced diabetes.

3.12.1 Animal housing and feeding conditions

Sprague-Dawley (SD) albino rats were procured from the Small animal breeding centre, College of Veterinary and Animal Sciences, Kerala Agricultural University, Mannuthy, Thrissur, Kerala. Swiss albino mice were procured from animal house of Nandha College of Pharmacy and Research Institute, Erode, TN, India. The animals were provided with adequate environmental conditions (temperature-24±2 °C; relative humidity-50±10% and 12:12 light:dark cycle) with the standard commercial pellets (M/s. Hindustan Lever Ltd, Mumbai, Maharashtra, India) and purified water ad libitum. All the experiments were performed with the permission from Institutional Animal Ethics Committee (688/2/C-CPCSEA) and were in accordance with the guidelines of CPCSEA.
3.12.2 Preparation of test drugs

The bark and empty pods (2 kg each) were extracted with 70% acetone as detailed in section 3.10.1 and dried at 40 °C. They were denoted as AAb for *A. auriculiformis* bark extract and AAep for *A. auriculiformis* empty pod extract. They were dissolved in 0.5% carboxy methyl cellulose (CMC) in (fixed dose in mg)/10 mL concentration and administered with the desired volumes according to the body weight of animals to the respective groups.

3.12.3 Acute toxicity studies

The healthy Swiss albino mice (20-25 g) fasted for 3-4 h (provided only with water) were randomly transferred to 5 groups (n=3/group). They were fed orally with extracts in the dose range of 5, 50, 300 and 2000 mg/kg body weight (b.w.) post oesophagus (p.o.) with the control of 0.5% CMC. The study was carried out as per OECD guidelines - 423 (Acute toxic class method) (OECD, 2001). The animals were observed for any sign of toxicity, morbidity and mortality for the first 24 h with the special attention during the first 4 h. They have also analyzed for the changes in behavioral, neurological and autonomic profile. Further, they were observed for a period of 72 h and till the completion of 14 days. Test dose was calculated as per Naskar *et al.* (2011).

3.13 Hepatoprotective activity

3.13.1 Experimental design and animal grouping

The SD rats weighing 100-150 g were used for the study. Animals were divided into 7 groups (n=6/group) as follows;

- GI - Normal control (NC) rats received distilled water 5 mL/kg b.w. p.o./7 days
- GII - Paracetamol control (PC) rats received distilled water 5 mL/kg b.w. p.o./7 days (except on 5th day)
- GIII - Rats received the standard drug silymarin 100 mg/kg b.w. p.o./7 days
- GIV - Rats received test drug 1 (AAb) - 200 mg/kg b.w. p.o./7 days
- GV - Rats received test drug 1 (AAb) - 400 mg/kg b.w. p.o./7 days
- GVI - Rats received test drug 2 (AAep) - 100 mg/kg b.w. p.o./7 days
- GVII - Rats received test drug 2 (AAep) - 200 mg/kg b.w. p.o./7 days
All the animals in the group (GIII-GVII) were pre-treated with their respective drugs for 5 consecutive days. On the fifth day of experimental period, after the drug administration of respective treatments, all the animals except those in GI were administered with paracetamol 2 g/kg b.w. p.o. On the seventh day, after 2 h of respective drug treatments, animals were anaesthetized using diethyl ether inhalation jar. Blood was collected through cardiac puncture and the serum was separated.

3.13.2 Determination of key liver function biochemical markers

Liver function biochemical markers such as alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total bilirubin and total protein has been evaluated in the serum obtained from the experimental animals according to the supplier’s specifications (Agappe Diagnostics Ltd, Ernakulum, Kerala, India; Accurex Biomedical Pvt Ltd, Thanae, Maharashtra, India) from the standard kits and the results were expressed as units/litre (U/L) or mg/dL.

3.13.3 Determination of key oxidative stress markers

One part of the liver tissue from the sacrificed experimental animals were washed and homogenised (1:10, w/v) in ice-cold 50 mM Tris buffer (pH 7.4). The contents were centrifuged at 10000 ×g for 20 min at 4°C and the supernatant obtained was analyzed for superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and glutathione peroxidase (GPx). Lipid peroxidation byproduct malondialdehyde (MDA) was measured in the form of thiobarbituric acid reactive substances (TBARS).

3.13.3.1 Lipid peroxidation

Lipid peroxidation (LPO) in the tissue homogenate was measured by estimating TBARS (Ohkawa et al., 1979). The tubes containing 1 mL of tissue homogenate was subsequently added with 1 mL of tris buffer (0.02M, pH 7.5), 1 mL of 10% TCA and 1.5 mL of thiobarbituric acid (1.5%). The reaction mixture was boiled for 15 min in boiling water bath and cooled to room temperature. The contents were centrifuged at 1000 ×g for 20 min and supernatant was collected. The absorbance of the supernatant was measured at 535 nm against reagent blank. TBARS was expressed as nmol MDA/mg protein.
3.13.3.2 Superoxide dismutase

Superoxide dismutase activity was measured using pyrogallol auto-oxidation in Tris buffer (50 mM, pH 7.0) (Marklund and Marklund, 1974). The enzyme activity was expressed as U/mg protein. 0.5 mL of liver homogenate was mixed with 0.25 mL of cold absolute ethanol and 0.15 mL of chloroform and kept under shaking condition for 15 min. The suspension was centrifuged for 15 min at 10000 × g and 0.5 mL of supernatant was used for the assay. The reaction mixture for auto-oxidation consists of 2 mL Tris-HCl buffer (pH 8.2), 0.5 mL of 2 mM pyrogallol and 2 mL of distilled water. Initially, the rate of pyrogallol auto-oxidation was determined at an interval of 1 min for 3 min. This was considered as 100% auto-oxidation.

The assay mixture for the SOD activity determination was prepared as follows; 2 mL of Tris-HCl buffer (pH 8.2), 0.5 mL of 2 mM pyrogallol, 0.5 mL aliquots of the enzyme preparation from liver homogenate and distilled water to give a final volume of 4.5 mL. The blank was prepared simultaneously, with 2 mL of Tris-HCl buffer (pH 8.2) and 2.5 mL of distilled water. The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme was noted. The color developed was read at 470 nm at an interval of 1 min for 3 min. The % inhibition in the auto-oxidation of pyrogallol in the presence of enzyme preparation was converted to units of inhibition.

3.13.3.3 Catalase

Catalase activity was measured using H$_2$O$_2$ as the substrate (Aebi, 1983). The reaction was initiated by the addition of 1 mL of phosphate buffer (0.01M, pH 7.1), 0.5 mL of H$_2$O$_2$ (0.2M) and 0.4 mL of distilled water successively to 0.5 mL of tissue homogenate. After 60 sec, reaction was stopped by the addition of 2 mL of dichromate-acetic acid reagent. The tubes were kept in a boiling water bath for 10 min and cooled. The absorbance of the chromophore developed was read at 620 nm. A system devoid of enzyme served as control. Activity of catalase was expressed as µmol of hydrogen peroxide consumed/min/mg protein.

3.13.3.4 Reduced glutathione

Liver homogenate of 1 mL was precipitated with 1 mL of 5% TCA. The precipitate was removed by centrifugation (1500 ×g for 20 min). An aliquot of 1 mL of the supernatant,
0.2 mL of Ellman’s reagent also called 5,5'-dithio-bis (2 nitro benzoic acid) (DTNB) reagent (0.6 mM) and 0.5 mL of phosphate buffer (0.2M, pH 8.0) were added to make the final volume of 3 mL. The reaction mixture was incubated for 20 min at room temperature and the absorbance was read at 412 nm against blank. The amount of glutathione is expressed as μg/mg protein (Moron et al., 1979).

3.13.3.5 Glutathione peroxidase

Glutathione peroxidase was measured according to the method of Rotruck et al. (1973) with some modifications. The reaction mixture consists of 0.4 mL of phosphate buffer (0.4M, pH 7.0), 0.1 mL of sodium azide (10 mM), 0.2 mL of reduced glutathione (3 mM), 0.1 mL of H₂O₂ (25 mM) and 1 mL of liver homogenate. The final volume of the reaction mixture was made up to 2 mL with distilled water and incubated at 37 °C for 60 sec. After incubation the reaction was terminated by the addition of 0.5 mL of TCA (5%). Then the contents were centrifuged at 1500 × g for 20 min. To determine the remaining glutathione content, the supernatant of 1 mL was taken and added with 3 mL of disodium hydrogen phosphate solution and 1 mL of Ellman’s reagent. The color developed was read at 412 nm against reagent blank prepared with disodium hydrogen phosphate solution (0.3M) and 1 mL of the DTNB reagent. The activity was expressed U/mg protein.

3.14 Anti-diabetic activity

3.14.1 Induction of experimental diabetes

Diabetes was induced in overnight fasted SD rats weighing 150-200 g by a single intraperitoneal injection of 150 mg/kg b.w. alloxan monohydrate dissolved in sterile distilled water. After injection, the rats were allowed to food and water ad libitum. The development of diabetes (hyperglycemia) was confirmed after 72 h. Animals with fasting blood glucose levels more than 200 mg/dL were considered as diabetic and selected for the study.

3.14.2 Experimental design and animal grouping

After the induction process, the diabetic rats were randomly divided into 6 groups along with one control group consists of non-diabetic rats (n=10/group). The test drugs and standard drug glibenclamide were administrated for a period of 14 days with the group allotment as follows,
GI - Non-diabetic control (NDC) rats received distilled water 5 mL/kg b.w. p.o./14 days
GII - Diabetic rats received distilled water 5 mL/kg b.w. p.o./14 days
GIII - Diabetic rats received reference drug glibenclamide 5 mg/kg b.w. p.o./14 days
GIV - Diabetic rats received test drug 1 (AAb) - 200 mg/kg b.w. p.o./14 days
GV - Diabetic rats received test drug 1 (AAb) - 400 mg/kg b.w. p.o./14 days
GVI - Diabetic rats received test drug 2 (AAep) - 100 mg/kg b.w. p.o./14 days
GVII - Diabetic rats received test drug 2 (AAep) - 200 mg/kg b.w. p.o./14 days

3.14.3 Biochemical analysis

Changes in fasting blood glucose levels (Glucometer - One-Touch Basic®, Lifescan, Inc 2001 Milpitas, CA, USA) and body weight of each group were determined weekly. On 15th day the animals were anaesthetized using diethyl ether inhalation jar, sacrificed and the blood was collected through cardiac puncture. From the serum obtained, the following biochemical parameters were estimated; total cholesterol (TC), triglycerides (TGs), high density lipoprotein cholesterol (HDL-C), urea and creatinine according to the supplier’s specifications (Accurex Biomedical Pvt Ltd, Thanae, Maharashtra, India) from the standard kits. Low density lipoprotein cholesterol (LDL-C) and Atherogenic index (AI) has been calculated using the following formula: LDL-C = TC - (HDL-C) - (TGs/5); AI = log (TGs/HDL-C) (Friedewald et al., 1972).

3.15 Statistical analysis

The data of in vitro antioxidant assays for bark and empty pod extracts were subjected to a one-way analysis of variance (ANOVA) and the significant difference between mean values was determined by Duncan’s multiple range test (P < 0.05) using the SPSS (Statistical Package for the Social Sciences) version 13.0. (SPSS Inc., Chicago, Illinois, USA). Values were expressed as mean of triplicate determinations±standard deviation (SD).

For in vivo analysis, the values were expressed as mean±SD (n=6). The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by post-hoc Dunnnett’s multiple comparison test using the SPSS (version 13.0). Significant difference were analyzed at 3 levels; P < 0.05 (significant), P < 0.01 (most significant) and P < 0.001 (highly significant).